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ARTIFICIAL INDUCTION OF POLYPLOIDY IN ORCHIDS BY THE USE OF COLCHICINE

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H. Y. Nakasone and H. Kamemoto

INTRODUCTION

Studies on chromosome numbers of plants have shown the occurrence of a large number of natural polyploids. Many plants selected from the wild for domestication were later found to be polyploids. They were selected because of specific phenotypic qualities even though their ploidy was unknown at the time of selection. These polyploids originated in a number of ways. Some occurred through spontaneous doubling of chromosomes in somatic tissues, while others were the result of nonreduction of gametes, polyspermy, or polyploidization of gametes. Many present-day advance generation hybrids are the results of hybridization of naturally occurring polyploids.

In the orchid industry, polyploidy has come to assume a prominent role in the production of superior types. Kamemoto (*21*, *22*) reported that many award-winning *Cattleya* hybrids were triploids and tetraploids. Storey (*38*, *39*) and Kamemoto (*23*, *24*) showed that superior hybrid selections in the vandaceous group were triploids, tetraploids, and pentaploids. Kosaki (*25*) and Ito and Matsuura (*19*) showed that a number of superior *Dendrobium* hybrids were triploids and tetraploids. Mehlquist showed the occurrence of polyploids in *Paphiopedilum* (*27*) and *Cymbidium* (*28*).

Because polyploid orchids have been established to be generally superior to their diploid counterparts, the artificial induction of polyploidy assumes particular significance. If somatic doubling can be induced at will with a chemical such as colchicine, superior horticultural varieties might be obtained immediately. Such induced tetraploids might be utilized in breeding further polyploids.

In the course of an extensive breeding program in orchids, a relatively high degree of sterility has been encountered among intergeneric hybrids and to a lesser degree among interspecific hybrids. The cytological basis for such sterility in orchids is largely nonhomology of the chromosomes (*21*, *22*, *23*, *38*, *39*). It has been well demonstrated in plants other than orchids that this type of sterility barrier could be removed by doubling the chromosome numbers of the sterile types by the use of colchicine. Restoration of fertility among the sterile hybrids was found desirable in order to advance the breeding program.

In accordance with these considerations, attempts were made to find practical methods for inducing polyploids in selected orchid groups by the use of colchicine. This investigation also entailed the study of the characteristics of the induced polyploids and comparing them with their diploid counterparts with respect to morphological differences.

REVIEW OF LITERATURE

The historical aspects of the use of colchicine in biology have been reviewed by Eigsti and Dustin (*15*). The highly efficient nature of colchicine in doubling the chromosome numbers has been unquestionably demonstrated by many investigators in fruit crops such as peaches, grapes, strawberries (*9, 10, 11, 12*); in floricultural crops such as zinnias, petunias, Easter lilies, cosmos (*5, 6, 16, 17, 31*); in vegetable crops such as tomatoes, muskmelons, lettuce (*2, 32, 36, 40*); and in a large number of field crops (*4, 14, 18, 30, 41*). Many of these induced polyploids were found to be superior to their diploid counterparts and have been introduced as commercial varieties.

In orchids several attempts to induce polyploidy with colchicine have been reported, but the results have not been as encouraging or successful as with other plants. The first report of induced polyploidy in orchids is by MacLeod (*26*) in 1947. He reported delayed flowering and two sizes of embryos in selfed seeds of treated flowers. In *Laelia anceps* he reported that the treated flowers were twice the normal size and had intensified color. Moore (*29*) in the same year reported on his work involving *Cattleya trianaei* var. *alba*. Leaves of some colchicine-treated plants were tough and wrinkled and the plant seemed to have increased its blooming capacity. Rotor (*33*) has reported considerable success in the treatment of a number of genera of orchids. However, in all of these cases achievement of polyploidy was assumed on morphological evidences and not from cytological studies.

MATERIALS AND METHODS

Materials

Colchicine—Colchicine, a poisonous alkaloid extracted from the seeds and corms of *Colchicum autumnale*, was used in various concentrations. It is highly soluble in water, a property desirable in application.

Plant materials—For convenience and ease of reference, the orchid materials used in the treatments are listed together with other pertinent information in table 1.

Nutrient agar—The nutrient agar used in the germination of treated and untreated seeds was formulated by Mr. J. P. Martin of the Experiment Station of the Hawaiian Sugar Planters' Association.

The formula is given as follows:

$Ca(NO_3)_2 \cdot H_2O$	1.00 gm.
KH_2PO_4	1.00 "
$MgSO_4 \cdot 7H_2O$	0.50 "
$(NH_4)_2SO_4$	0.25 "
NH_4NO_3	0.25 "
Tap water	1000.00 ml.
Peptone	5.00 gm.
Sucrose	20.00 "
Agar	12.00 "

$FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$—0.7 gm. per 100 ml. of water as stock solution. 2 ml. of this solution is added to a liter of nutrient agar to satisfy the iron requirement.

TABLE 1. List of plant materials used in the experiments

NAME	CROSS	LEAF TYPE	PARTS OF PLANTS USED	CHROMOSOME NUMBER (2n)
Vanda Miss Joaquim	*V. teres* × *V. hookeriana*	terete	tip cutting young shoot flower spike seed	38
V. Michael Beaumont	*V. Manila* × *V. Roeblingiana*	spatulate	mature plant	38
V. Princess Elizabeth	*V. hookeriana* × *V. sanderiana*	semi-terete	tip cutting	38
V. Fair Queen	*V. Clara Fisher* × *V. Rothschildiana*	spatulate	seed	
	V. Ellen Noa × *V. Bill Sutton*	spatulate	seed	
Dendrobium Molokai	*D. Colin Potter* × *D. gouldii*		young shoot	
D. undulatum	species		seed	38
D. undulatum var. *Bromfieldii*	species		seed	38

Methods of Preparing Colchicine in Various Carriers

Solution method

a. Aqueous solution—Colchicine was weighed out in required amounts and dissolved in tap water.

b. Glycerine-colchicine solution—Required amounts of colchicine were dissolved in the following mixture, modified after Dermen (*10*): glycerine—16.5 ml., water—5.5 ml., and 10 percent solution of Triton B-1956—3 ml. The last ingredient is a sticker and spreading compound. According to Dermen, glycerine provides a nonvolatile medium which keeps the treated area moist and holds the colchicine in place to be absorbed gradually.

Lanolin paste method—Measured amounts of lanolin were melted (ca. 55° C.) and the required amounts of colchicine were added to the melted lanolin, stirred until completely dissolved, and left to solidify. This material was applied to plant parts with a toothpick.

Colchicine-nutrient agar—The inorganic salts were first dissolved in 1 liter of tap water. To this was added 2 ml. of ferrous ammonium sulfate from the stock solution and the entire content was heated to boiling. Peptone, sugar, and agar were then added with constant stirring. The pH was adjusted to approximately 5.2 and the solution poured into 250-ml. erlenmeyer flasks in 100-ml. proportions. To these flasks required amounts of colchicine were added and stirred until dissolved. Each flask was plugged with a cotton stopper and placed in an autoclave for sterilization at 15-pound pressure for 15 minutes. After sterilization, the flasks were left at room temperature to cool and solidify.

Methods of Colchicine Treatment

Treatment of seeds and protocorms—Seeds of *Vanda* and *Dendrobium* plants were either pre-soaked in aqueous solutions of colchicine and sowed in nutrient agar or sowed without pre-treatment into colchicine-incorporated nutrient agar. Aqueous-colchicine concentrations for pre-treatment ranged from 0.05 to 1.0 percent and for soaking durations of 1 to 10 days. Control lots were soaked in water. Concentrations of colchicine in nutrient agar ranged from 0.01 to 1.5 percent. All seeds were sterilized in diluted "Clorox"[1] solution (1:50) for 15 to 20 minutes before sowing.

In the case of protocorm treatments, seeds were first sown and germinated in nutrient agar. When green protocorms were formed, approximately 0.6 cc. of sterilized aqueous-colchicine was pipetted into each flask under aseptic condition. Colchicine solution was swirled around in the flask each day to wet the protocorms. Concentrations ranged from 0.01 to 1.5 percent. In all above treatments, several series were run to allow for contaminations.

When seedlings were approximately 1.5 to 2 cm. high with sufficient roots and leaves, they were removed from the flasks and transplanted into flats containing disinfected peatmoss as the planting medium. When plants were approximately 10 months old with good root system, chromosome counts were made from root-tip squashes.

Treatment of seedlings—*Dendrobium* seedlings, 1 to 3 cm. tall, were soaked in aqueous-colchicine ranging in concentration from 0.01 to 2.0 percent under two methods. The first method involved the immersion of seedlings for 3 hours in vials containing colchicine solution. The second method, designated as infiltration method after Braak and Zeilinga (*3*), consisted of immersing the seedlings in vials containing colchicine solution and placing the vials in an exsiccator in which a vacuum was created by means of a water vacuum pump. In 7 minutes the solution began to bubble. At that point, the pump was stopped and the plants were allowed to remain in vacuum for 10 minutes. This procedure is supposed to evacuate the air from the plants and allow the solution to penetrate the tissues more readily than by soaking without the vacuum. At the end of the treatment period for both methods, plants were removed from the vials, washed in tap water, and planted in peatmoss flats for later chromosome counts.

Treatment of inflorescence, cuttings, young shoots, and apical meristems of mature plants

a. *Treatment of inflorescence*—Flower spikes of *V. Miss Joaquim*, 2.5 to 20 cm. long, were selected and the apical section of the spikes was covered with absorbent cotton saturated with aqueous-colchicine solution ranging in concentration from 0.1 to 2.0 percent. Polyethylene bags were placed over the cotton to prevent drying. Duration of treatments extended from 8 hours to 5 days. At the end of the treatments, the cotton applicators were removed and the spikes were left to develop. Whenever possible, bud material from these treated spikes was used to determine ploidy.

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[1]"Clorox"—Commercial disinfectant containing 5.25 percent dry weight of sodium hypochlorite as active ingredient.

b. *Treatment of tip cuttings of mature plants*—Tip cuttings of *V. Miss Joaquim* and *V. Princess Elizabeth*, approximately 8 to 10 inches long, were made and the basal ends were immersed in vials containing aqueous solutions of colchicine. Concentrations ranged from 0.1 to 2 percent and the duration of treatments ranged from 1 to 20 days. At the end of the treatment period, cuttings were planted in cutting boxes containing wood shavings as medium. Root tips for chromosome counts were taken from roots which developed on new growth above the original treated apices. In some instances, apical growth after treatment was arrested for prolonged periods and axillary shoots emerged from the first or second node below the treated apex. In these cases, root tips were taken from the axillary growth. In a few cases, chromosome counts were made from bud marerials.

c. *Treatment of tip cuttings with young flower spikes*—Treatment method is identical to that of treatment b above except for the cuttings of *V. Miss Joaquim* possessing young flower spikes.

d. *Treatment of young shoots*—Young shoots of *V. Miss Joaquim*, 3 to 4 inches long, were either excised at the base or with part of the parent stem still attached to the base of the young shoots. The base of these shoots or of the mature stems was immersed in colchicine solutions of various concentrations for different durations. In the case of *V. Princess Elizabeth*, cuttings 6 to 8 inches long, each with a young lateral shoot near the terminal end, were used. At the end of the treatment period, each cutting was attached to a tree-fern pole and planted in a 5-inch clay pot for growth and further observations.

In the case of *D. Molokai*, shoots 3 to 8 inches long with roots 2 to 5 mm. long, were excised from the parent plants and the basal end immersed in aqueous-colchicine solutions.

e. *Treatment of vigorously growing plants*—Cuttings of *V. Miss Joaquim* and *V. Princess Elizabeth* were attached to tree-fern poles and planted in 5-inch clay pots several months before treatment time. When these plants were well established on the tree-fern poles, the lower part of the stem approximately 4 inches above the base of the plant was cut off and the newly cut base was immersed in aqueous-colchicine solution. This experiment was based on the theory that plants in vigorous and uninterrupted state of growth would have a greater absorbing capacity than cuttings removed from plants and interrupted in their growth processes.

f. *Treatment by incision of apical area*—The apical region of *V. Miss Joaquim* and *V. Michael Beaumont* was incised longitudinally to expose the apical meristem, and colchicine-lanolin paste ranging in concentration from 0.1 to 5.0 percent was administered into the incision with a toothpick (see figs. 6, 11, and 12). Controls were treated with lanolin paste without colchicine.

In a similar experiment, instead of using colchicine-lanolin paste, glycerine-colchicine solution was applied into the incisions with a camel-hair brush. To account for the possible effects of time of exposure to the colchicine and the possible drying of the solution, colchicine-glycerine solution was applied 2, 4, and 6 times, each application being given at 2-day intervals.

g. *Treatment by injection of colchicine*—The apical regions of *V. Michael Beaumont* (spatulate-leaf type) and young shoots (3 to 15 inches long) of *D. Molokai* were

treated by injecting aqueous-colchicine or glycerine-colchicine solution with capillary pipettes. These pipettes were made by stretching glass tubing to a thin point. In the case of *V. Michael Beaumont*, excess terminal leaves were cut off to facilitate the penetration of the pipette into the meristematic region between the folds of the leaf sheaths.

In one of the above experiments, the capillary pipettes were inserted and left in the plant for gradual release of the solution. In the second experiment, 3 to 6 drops of colchicine solution were injected and the pipette was removed immediately. Injections were given 1, 3, and 6 times at 3-day intervals. Colchicine concentrations ranged from 0.1 to 2.0 percent.

Method of Taking Data

a. *Visual effects on plants*—All plants were examined periodically to observe changes in appearance of the plant parts treated.

b. *Techniques in chromosome counts*—Root tips were cut and pre-treated in 0.002 M 8-oxyquinoline for 2 to 4 hours at approximately 40° to 45° F. and fixed in a modified Carnoy's fluid for 10 or more hours. Following fixation, roots were hydrolized in a 1:1 mixture of concentrated hydrochloric acid and 95 percent ethyl alcohol for 10 minutes and washed in water. Roots were then placed on slides in a drop of 1 percent aceto-orcein in 45 percent acetic acid and squashed. Slide preparation was done according to standard procedure for temporary mounts.

When bud material was available from treated plants or inflorescence, it was killed, fixed, hydrolyzed, and squashed in the same manner above.

c. *Paraffin sections for comparison of cell size between polyploid and diploid tissues*—Root tip and shoot apex regions were killed and fixed in FAA (formalin—5 cc., acetic acid—5 cc., 70 percent ethyl alcohol—90 cc.) for 18 hours or longer and dehydrated in tertiary butyl alcohol series, followed by infiltration with Parowax and tissue mat according to the standard paraffin method outlined by Johansen (*20*). Embedded materials were microtomed at 13 microns and stained with safranin-fast green.

d. *Number and size of stomata*—Pieces of epidermal tissue were peeled from the leaves of diploid and polyploid plants and placed on the slide in several drops of water. Each leaf was divided into four sections, e.g., tip, median, base, and sheath. For each section, ten microscopic fields constituted one replicate, and this was replicated four times. Variance analysis as outlined by Snedecor (*37*) was employed in determining significance of difference between means of number and size of stomata on polyploid and diploid leaves. Where variance analysis showed significance between several means, Duncan's (*13*) multiple range and multiple F test was employed and the means are presented in ranked order.

EXPERIMENTAL RESULTS

Treatment of Seeds and Seedlings

a. *Treatment of seeds before sowing*—An effective method reported by others (*1*, *7*, *40*) is the soaking of seeds in aqueous solutions of colchicine for several

hours to a few days before planting. To determine whether orchid seeds would respond to such treatments, seeds of several species and hybrids of *Vanda* and *Dendrobium* were treated according to methods described in the previous section and sowed in nutrient agar.

It was found that at all concentrations and for durations up to 4 days, germination was good. Beyond 4 days, even concentrations as low as 0.1 percent affected germination and post-germination mortality. Survivors in most instances were stunted and made little or no growth. In most instances, protocorms were highly proliferated. Many succumbed through latent contaminations or died after transplanting into seedling flats. At 1.0 percent and for durations of 8 to 10 days, protocorms enlarged without differentiation. Only the control (seeds soaked in water) and seeds treated with 0.1 and 0.5 percent colchicine for durations up to 5 days resulted in survival of seedlings. Chromosome counts from root-tip smears showed the survivors to be diploids with 2n = 38 chromosomes.

b. *Treatment by sowing seeds in colchicine agar*—Seeds of *Vanda* and *Dendrobium* were sterilized and sown directly on colchicine-incorporated nutrient agar. Germination was good in concentrations ranging from 0.01 to 0.5 percent but above these concentrations, germination rate was low and post-germination mortality was high. Excessive proliferation of protocorms was noted in concentrations above 0.5 percent. Surviving plants in concentrations below 0.3 percent were determined to be diploids.

c. *Treatment of protocorms*—Since colchicine is reported to be most effective upon plant tissues in highly active state of growth, seeds in protocorm stage of germination were subjected to various concentrations of colchicine. In the low concentrations (0.01 to 0.05 percent) protocorms continued their development and differentiation as in the control lots. From 0.1 percent and up, protocorm mortality increased. A number of surviving protocorms became enlarged and further differentiation was arrested. Only plants from the checks, 0.01, 0.05, and few of the 0.1 percent treatments, survived transplanting. These were all determined to be diploids.

Dendrobium seedlings, 1 to 3 cm. tall, were soaked in aqueous-colchicine with and without vacuum as described under Methods. The results showed that seedlings are very sensitive, especially to concentrations beyond 0.1 percent, even without vacuum. Mortality for the nonvacuum treatment was approximately 50 percent in concentrations less than 1.0 percent and 100.0 percent mortality in concentrations above 1.5 percent. Mortality of the vacuum-treated plants in low concentrations was much higher than the plants treated without vacuum. Surviving plants were all determined to be diploids.

Treatment of Young Developing Flower Spikes

A useful method, if successful, is the treatment of young flower spikes to induce the production of 2n pollen grains. These 2n pollen grains could be utilized in pollination of appropriate types to produce 4n and 3n progenies.

With this in mind, young spikes of *V. Miss Joaquim* were treated according to methods described previously. At concentrations of 0.1 and 0.5 percent and for

durations up to 2 days, the effects were mild. Some swelling occurred and the first few maturing buds turned yellow and abscised. As duration of exposure or concentration was increased, the spikes became distorted and buds turned yellow and abscised at an early stage. Even at 0.1 percent for durations beyond 3 days, spikes and buds were distorted and further development was arrested. At 1.0 percent and for durations up to 2 days, early buds abscised but the later ones were available for chromosome counts. Considerable distortion was noted. In all cases no doubling of chromosomes was observed.

Treatment of Mature Plants and Shoots

a. *Treatment of tip cuttings*—Tip cuttings of *V. Miss Joaquim* were treated as described under Methods and a summary of data from several similar trials is presented in table 2. The data show that the apex swelled and growth was temporarily arrested but new growth usually resumed. (Figures 1 and 2 show swelling of the apical region, while figure 3 shows swelling of aerial roots.) At concentrations above 3.0 percent, exposure for 1 day was sufficient to cause rather severe damage to the cuttings. Plants shrivelled and remained dormant for as long as 2 years and in most instances did not recover. This is illustrated in figures 4 and 5. Low concentrations for long durations had the same detrimental effect. Occasionally, a plant recovered and grew when treated with 0.5 to 1.0 percent for as long as 15 days. Figure 8 shows a plant which recovered after a long period of inactivity. Chromosome counts were made from root tips emerging from new growth beyond the treated apex. Tetraploid counts were observed in one plant treated with 0.5 percent for 2 days and two plants treated with 1.0 percent for 2 days. Treatment with 0.5 percent for 4 days resulted in another tetraploid. In all, there were four tetraploid plants. Doubling occurred at concentrations of 0.5 to 1.0 percent for treatment time of 2 to 4 days.

b. *Treatment of tip cuttings with young flower spikes*—In a similar experiment, tip cuttings of *V. Miss Joaquim* possessing young developing flower spikes were selected. The purpose of selecting this type of material was to determine whether colchicine could be translocated to the inflorescence apex to effect doubling in the pollen mother cells as well as in the terminal vegetative apex.

Colchicine effects on the spikes were similar to those observed in the direct treatment of the spikes. At concentrations of 0.5 and 1.0 percent for treatment time of 2 and 4 days, spike apices were deformed and swollen. Figure 7 shows a set of three plants with affected spikes. The plant to the extreme left was treated with 0.5 percent aqueous-colchicine for 4 days and shows curvature of the spikes. The two plants in the middle show spikes which are beginning to swell. The plant to the extreme right is the control with normal spike. This photo was taken 13 days after treatment. No diploid spores were found.

The effects on the shoot apex were similar to those obtained in the previous experiment. Out of a total of 45 plants, two tetraploids were found—one resulting from treatment with 1.0 percent colchicine for 4 days and the other with 0.5 percent colchicine for 6 days.

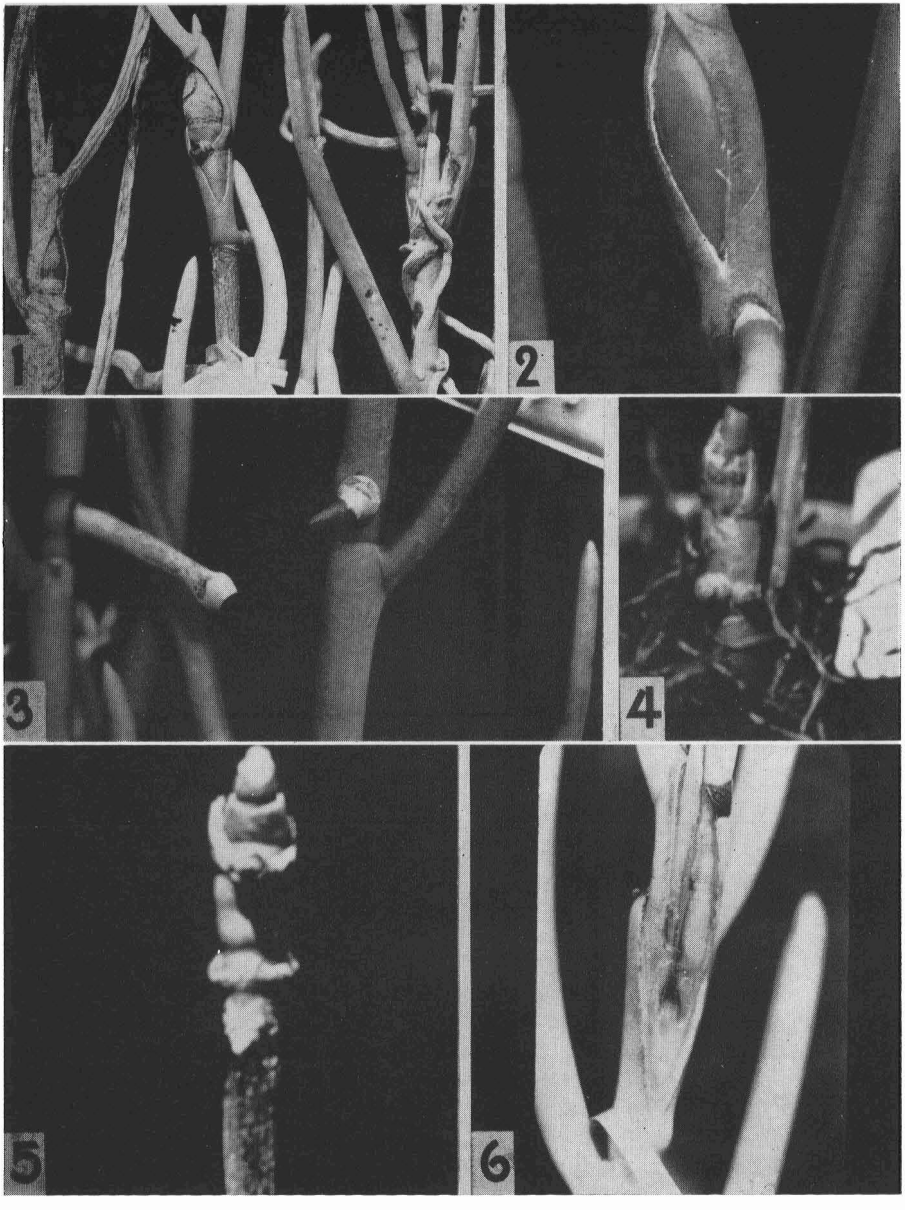

PLATE I

FIGURE 1. Aqueous-colchicine treated *V. Miss Joaquim* cuttings showing swelling of apex. *Left*, 4.0%—1 day; *center*, 3.0%—1 day; *right*, 0.5%—20 days.
FIGURE 2. Splitting of leaf sheath caused by swelling of apex.
FIGURE 3. Swelling of roots one month after treatment.
FIGURE 4. Severe effects by high concentration. Defoliation and long dormancy.
FIGURE 5. Severe effects by long duration of treatment.
FIGURE 6. Incision method using lanolin-colchicine paste.

TABLE 2. Results of immersing basal ends of *V. Miss Joaquim* tip cuttings in colchicine solution. (Summary of several treatments.)

CONCENTRATION PERCENT	DURATION DAYS	NO. OF PLANTS	SYMPTOMS AFTER TREATMENT*	NO. DEAD†	PLOIDY OF SURVIVORS‡
0.5	1	5	no visual effect	0	diploid (2n = 38)
1.0	1	5	apex, roots swollen	4	"
1.5	1	5	apex, roots swollen	2	"
2.0	1	5	stems, leaves shrivelled	4	no growth (no count)
3.0	1	5	shrivelled	3	" " " "
5.0	1	5	shrivelled	5	" " " "
0.5	2	5	apex swollen	0	1—tetraploid (4n = 76)
1.0	2	5	apex swollen	3	2—tetraploids
1.5	2	5	apex swollen, leaves shrivelled	2	diploid
2.0	2	5	shrivelled	3	1—no growth 1—diploid
3.0	2	5	shrivelled	2	no growth (no count)
5.0	2	5	shrivelled	5	" " " "
1.0	3	4	apex swollen, leaves shrivelled	0	diploid
3.0	3	4	apex swollen, leaves shrivelled	1	2—diploids 1—no growth
5.0	3	4	apex swollen, leaves shrivelled	1	3—no growth
0.5	4	6	apex swollen	0	5—diploids 1—tetraploid
1.0	4	6	apex swollen	0	2—no growth 4—diploids
1.5	4	6	apex swollen, leaves shrivelled	1	diploid
2.0	4	6	apex swollen, leaves shrivelled	1	no growth
0.5	5	5	apex swollen, leaves shrivelled	1	diploid
1.0	5	5	apex swollen, leaves shrivelled	2	diploid

(*Continued*)

*Observed 2 to 3 months after treatment.
†Data taken 6 months to 2 years after treatment.
‡In some cases where no growth resulted from the apex, ploidy given is from new shoot arising immediately below apical region.

TABLE 2. *Continued*

CONCENTRATION PERCENT	DURATION DAYS	NO. OF PLANTS	SYMPTOMS AFTER TREATMENT*	NO. DEAD†	PLOIDY OF SURVIVORS‡
0.5	6	8	apex swollen, leaves shrivelled	0	diploid
1.0	6	8	apex swollen, leaves shrivelled	2	4—no growth (no count) 2—diploids
1.5	6	8	plants shrivelled	4	1—diploid 3—no growth
2.0	6	8	plants shrivelled	3	5—no growth
0.5	10	6	plants shrivelled	4	no growth
1.0	10	6	plants shrivelled	3	diploid
1.5	10	6	plants shrivelled	4	no growth
2.0	10	6	plants shrivelled	5	no growth
3.0	10	6	plants shrivelled	4	no growth
0.5	13	3	plants shrivelled	0	1—diploid 2—no growth
0.5	15	3	plants shrivelled	1	1—diploid 1—no growth
1.0	15	3	plants shrivelled	2	diploid
0.5	20	3	plants shrivelled	1	no growth

*Observed 2 to 3 months after treatment.
†Data taken 6 months to 2 years after treatment.
‡In some cases where no growth resulted from the apex, ploidy given is from new shoot arising immediately below apical region.

Treatment of Young Shoots

a. *Treatment of young shoots excised from mature plants*—On the assumption that young, rapidly growing shoots would be more responsive to colchicine treatment, young shoots of *V. Miss Joaquim*, *V. Princess Elizabeth*, and *D. Molokai* were excised from mature plants and were treated by immersing the basal ends in aqueous-colchicine solutions. Data for shoots of *V. Miss Joaquim* are presented in table 3.

Young shoots appeared to be more sensitive to colchicine than tip cuttings of mature plants. Concentrations as low as 0.1 percent for 2 days effected considerable swelling at the apex with a long delay in recovery. Most of the shoots treated for 6 days made no further growth from the affected apices. Among those that recovered and grew, one tetraploid was found in treatment 0.5 percent for 4 days. This plant, shown in figure 10, produced two new shoots from the swollen area and one shoot

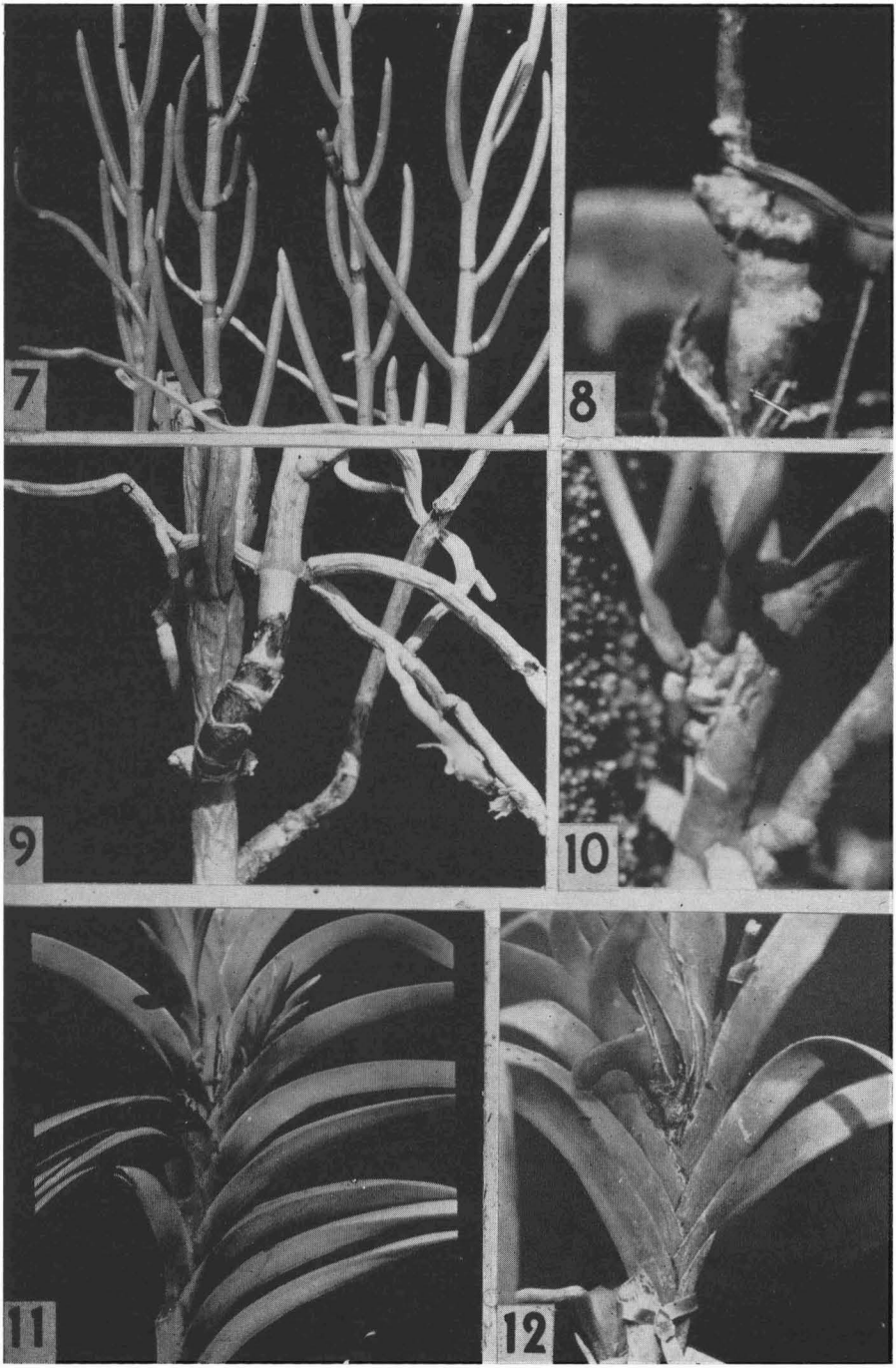

PLATE II

TABLE 3. Results of immersing basal ends of young shoots of *V. Miss Joaquim* in aqueous-colchicine solution

CONCENTRATION PERCENT	DURATION DAYS	NO. OF PLANTS	SYMPTOMS AFTER TREATMENT	NO. DEAD	PLOIDY OF SURVIVORS
0.1	2	6	apex swollen, growth delayed	0	diploid (2n = 38)
0.5	2	6	apex swollen, growth delayed, leaves shrivelled	2	"
1.0	2	6	leaves, stems shrivelled	4	"
0.1	4	6	apex swollen	0	diploid
0.5	4	6	apex swollen, leaves shrivelled	2	3—diploids 1—tetraploid (4n = 76)
1.0	4	6	plant shrivelled	2	diploid
0.1	6	6	apex swollen	0	2—no growth 4—diploids
0.5	6	6	apex swollen, leaves shrivelled	1	3—no growth 2—diploids
1.0	6	6	apex swollen, leaves shrivelled	2	2—no growth 2—diploids

at the next lower node. Chromosome counts from root tips showed that only the shoot formed below the swelling (designated as shoot No. 3) was tetraploid.

In the case of *V. Princess Elizabeth*, only two plants (0.1 percent for 4- and 6-day treatments) survived but no new growth was produced. Shoots of *D. Molokai* were very sensitive to treatments. Early yellowing of leaves and defoliation were observed, and within 3 months, all plants in the 4-day duration involving all concentrations were dead. Most of the plants receiving treatments above 1.0 percent were dead regardless of duration of treatment. Chromosome determinations were not made since none of the survivors produced any flowers.

b. *Treatment of young shoots attached to mature stem sections*—Since immature tissues of young shoots were too sensitive to colchicine, shoots of *V. Miss Joaquim* and *V. Princess Elizabeth* were removed with sections of mature stem of the parent plant still attached. The bases of the mature stems were immersed in colchicine solution. Post-treatment symptoms were similar to those obtained in the previous

PLATE II

FIGURE 7. *V. Miss Joaquim* cuttings with flower spikes affected by colchicine. Cutting on the extreme right is the control (13 days after treatment).
FIGURE 8. New shoot development from severely affected *V. Miss Joaquim* apex.
FIGURE 9. Colchicine effect on a young shoot just emerging when the cutting was treated.
FIGURE 10. Two new shoots from the swelling on the left and one shoot below the swelling of *V. Miss Joaquim* cutting.
FIGURE 11. Two shoots emerging from incised apex of *V. Michael Beaumont* treated with lanolin-colchicine paste.
FIGURE 12. Shoot development from within the incised area of *V. Michael Beaumont*.

experiment with young shoots, but the mortality rates were much lower. *V. Princess Elizabeth* again showed greater sensitivity to treatment. In most instances terminal growth was arrested for a long time. Ploidy determinations among the survivors showed that doubling occurred in one shoot of *V. Miss Joaquim* receiving 0.5 percent treatment for 3 days. In one plant of *V. Princess Elizabeth* treated with 1.5 percent for 4 days, initial count from the first root showed tetraploidy but subsequent counts from the same root which regenerated new tips and counts from other roots showed diploidy. This seems to be a case of mixoploid condition which will be discussed in another section.

c. *Treatment of plants well established and in vigorous state of growth*—In this experiment well-established, rooted tip cuttings of *V. Miss Joaquim* were used. This method allowed treatment of plants with least disturbance in their growth processes. The lower part of the stem, approximately two nodes above the base, was cut in each case and freshly cut bases were immersed in colchicine solution. External symptoms of the apex were similar to other treatments already discussed. In some cases growth of the treated apices was more or less permanently arrested and new shoots emerged from nodes below the swelling. Chromosome counts from shoots emerging below the swelling showed only diploidy. Among the survivors that grew from the apex, only one plant treated with 1.0 percent for 4 days showed doubling.

d. *Treatment of plants by incision method using colchicine-lanolin paste*—Treatment methods are described in detail under Methods and the incised plants are shown in figures 6, 11, and 12. Concentrations ranged from 0.1 to 5.0 percent with 0.5 percent intervals. In the case of *V. Michael Beaumont*, growth was retarded by 1.5 percent colchicine, while *V. Miss Joaquim* was retarded by concentrations as low as 0.5 percent. In most instances growth was arrested temporarily and chromosome counts were made from subsequent growth either from the treated apices or from new shoots emerging from within the incised area. Results of these trials were completely negative.

In a similar trial involving incisions, glycerine-colchicine solution (0.1, 0.5, and 1.0 percent solution) was used instead of the lanolin preparation. Since the plants were grown on nursery benches in direct sunlight, glycerine-colchicine was applied 2, 4, and 6 times to allow for possible drying. In all cases some swelling occurred at the incised areas but cytological studies from root tips of subsequent growth showed no doubling of chromosomes. Where incised terminals failed to grow, secondary shoots were examined. These were all found to be diploid.

e. *Treatment by injection of colchicine solution*—Aqueous and glycerine-colchicine solutions were injected into the apical meristem by means of fine capillary pipettes. The results of glycerine-colchicine treatment were similar to that reported here for the aqueous solution.

In the case of *D. Molokai*, the apices of all treated plants died, indicating that treatment was too severe.

The results for *V. Michael Beaumont* were quite variable as far as effects on growth were concerned. Even the control plants with water injected into the apex failed to

TABLE 4. Summary and evaluation of induced tetraploids

TREATED MATERIAL	TREATMENT METHOD	NATURE OF PLOIDY*
tip cuttings	0.5%—2 days	temporary
tip cuttings	0.5%—4 days	temporary
tip cuttings	0.5%—6 days	temporary
tip cuttings	1.0%—4 days	temporary
tip cuttings	1.0%—2 days	temporary
tip cuttings	1.0%—6 days	continuing
tip cuttings	1.5%—4 days	continuing
young shoot excised	0.5%—4 days	pure, continuing
young shoot on old stem	0.5%—3 days	temporary
cutting established on tree-fern pole	1.0%—4 days	temporary

**Temporary* indicates that tetraploidy was noted in the first few observations but not found in subsequent counts.
Continuing indicates presence of 4n cells although mixed with 2n cells.
Pure indicates only 4n cells.

grow. This suggests injury to the apical bud by the capillary tubing rather than by direct effects of the substance injected. Chromosome counts for the surviving plants showed no doubling. In several cases pollen mother cells were examined and all were found to be diploid with 19 pairs of chromosomes at metaphase I.

Evaluation of Methods and the Nature of the Induced Polyploids

A summary of all induced polyploids and the methods by which they were obtained is presented in table 4. The largest number of polyploids was obtained from treatment of tip cuttings of *V. Miss Joaquim*. Young shoots and vigorously growing plants of *V. Miss Joaquim* were also responsive but not to any great extent. The method of colchicine application in all of these cases was by immersing the basal ends into colchicine solution. None of the other methods employed was effective.

The concentration and duration of exposure to colchicine presented in the second column of this table indicate that concentrations between 0.5 and 1.0 percent were most effective in doubling the chromosome number. Only in one case 1.5 percent was effective. The duration of treatment was confined to a range between 2 and 6 days. Durations less than 2 days or longer than 6 days were not effective at any of the concentrations used.

The nature of the polyploids obtained is very interesting. In seven out of ten polyploids obtained, continued chromosome counts from successive roots showed that doubling was temporary. After the first few counts showing tetraploidy, complete reversion to diploidy occurred.

In one case doubling of a shoot appeared to be complete since no diploid cells were found in the examination of root tips at several nodes. This shoot arose through treatment of a *V. Miss Joaquim* cutting with 0.5 percent aqueous-colchicine for 4 days and is shown in figure 10. The lower shoot on the right is the tetraploid shoot, while the upper two shoots emerging from the swollen apex are diploids.

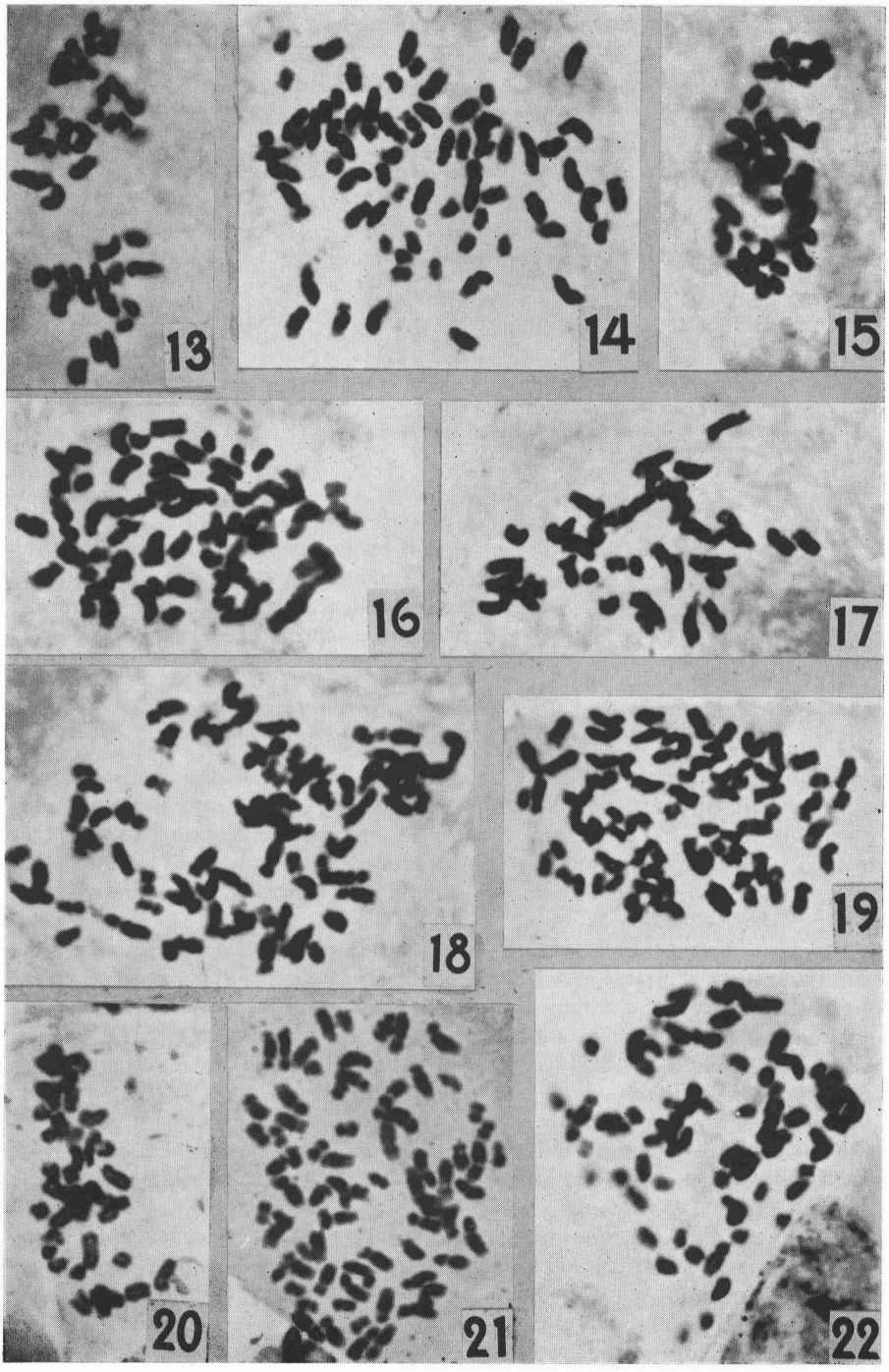

PLATE III

In two cases, tetraploidy has been maintained although diploid cells have been observed at various nodes. Figures 13 to 18 show chromosome figures at the various nodes of plant 1.0/6 (1.0 percent colchicine for 6 days). Figure 13 shows a diploid count at node 17, while figure 14 shows a tetraploid count at node 19. Figures 15 and 16 show diploid and tetraploid cells, respectively, in the same root tip at node 20. Two or even three roots appearing at different positions on the same node may differ in their chromosome counts. This is shown in figures 17 and 18, which represent roots A and B at node 5. Root A shows diploidy and root B, tetraploidy. Figures 19 to 22 show chromosome figures for different root tips at the various nodes of plant 1.5/4. Here again, nodes 6, 8, and 11 are tetraploids while node 7 is diploid.

Morphological Differences between Diploids and Colchicine-induced Polyploids

Photomicrographs of root and apical sections of tetraploid and diploid plants are presented to illustrate differences in size of cells. Figures 23 and 24 show the root apices of diploid and tetraploid plants, respectively. Cells in figure 24 appear to be much larger than those found in figure 23. These photomicrographs were all taken at the same magnification (177×).

A more well-defined difference in cell size may be seen in figures 25 to 28. Figures 25 and 26 show photomicrographs of tetraploid and diploid shoot meristems, respectively. It is clear that the cells composing the tunica and corpus layers in figure 25 are larger than those of comparable areas in figure 26. Similar difference in size of cells is also noted in figures 27 and 28, tetraploid and diploid, respectively, representing apical sections just approaching the apical dome region. In the actual slide preparation from which figure 25 was taken, approximate tetraploid counts of chromosomes were made in two cells, confirming the tetraploidy of that shoot.

To determine differences in number and size of stomata, counts and measurements were made from leaf samples of the diploid shoot No. 2, and the tetraploid shoot No. 3 of plant 0.5/4. Variance analysis for the mean number of stomata per microscopic field (7.16 and 7.03 stomata for 2n and 4n leaves, respectively) indicated no real difference.

The difference in the mean number of stomata for the three positions on the leaf (base, center, and tip) was highly significant. The means are presented in

PLATE III

(Chromosome figures magnified 2160×)

FIGURE 13. Diploidy in root A, node 17 of plant 1.0/6.
FIGURE 14. Tetraploidy in root A, node 19 of the same plant.
FIGURE 15. Diploidy in root A, node 20 of the same plant.
FIGURE 16. Tetraploidy in the same root and node of figure 15.
FIGURE 17. Diploidy in root A, node 5 of the same plant.
FIGURE 18. Tetraploidy in root B, node 5 of the same plant.
FIGURE 19. Tetraploidy at root B, node 6 of plant 1.5/4.
FIGURE 20. Diploidy at root A, node 7 of plant 1.5/4.
FIGURE 21. Tetraploidy at root A, node 8 of plant 1.5/4.
FIGURE 22. Tetraploidy at root B, node 11 of plant 1.5/4.

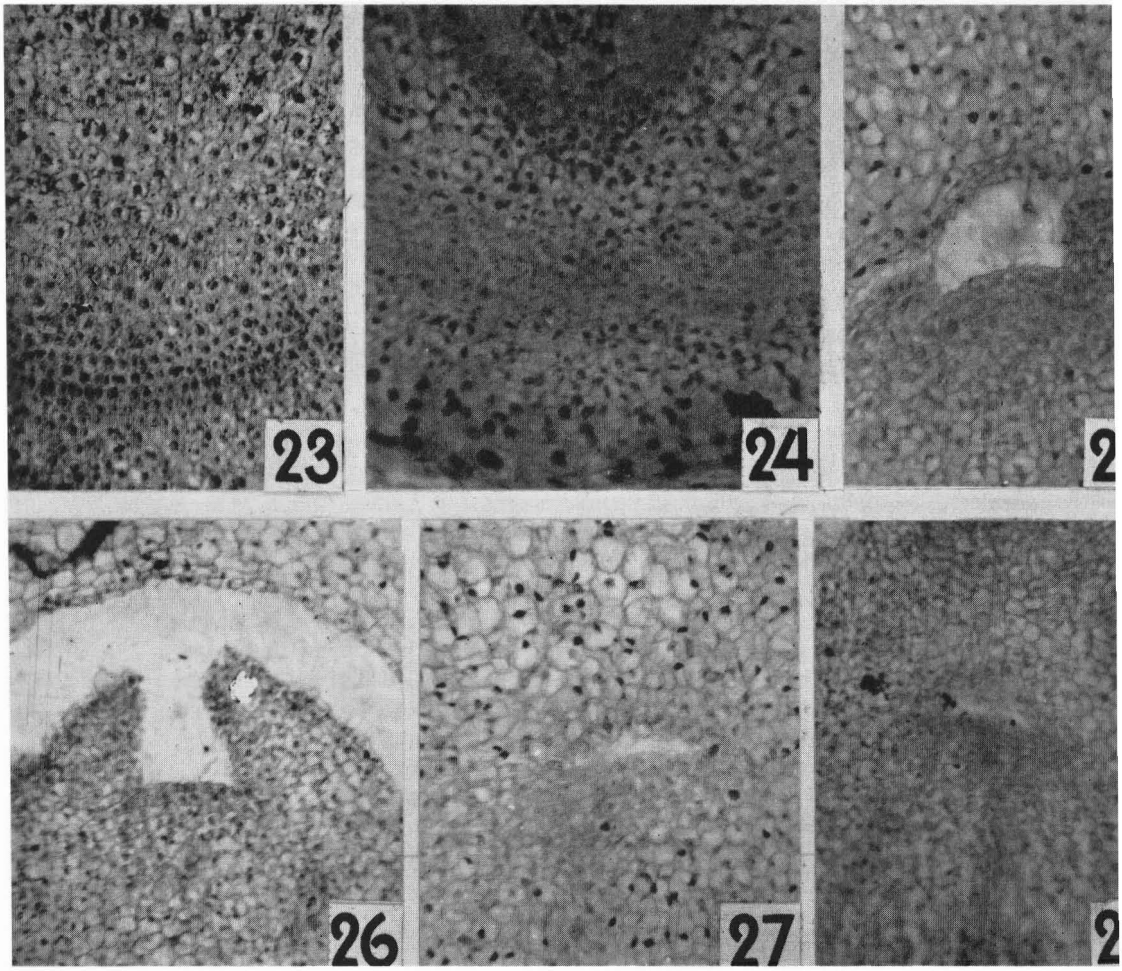

PLATE IV

FIGURE 23. Root apex of diploid plant showing normal cell size. Magnification at 177×.
FIGURE 24. Root apex of 4n plant (0.5/4 days) showing larger cell development (177× mag.).
FIGURE 25. Shoot apex of 4n plant (0.5/4) showing large cell development (177× mag.).
FIGURE 26. Shoot apex of 2n plant showing normal cell development (177× mag.).
FIGURE 27. 4n shoot apex several sections away from the apical dome, showing relatively large cells (177× mag.).
FIGURE 28. 2n shoot apex at comparable area showing smaller cells (177× mag.).

table 5 in ranked order according to Duncan's (*13*) method. These means represent the grand means of 2n and 4n leaves combined since there was no real difference between ploidy. The number of stomata increased from base to the tip of the leaf. Variance analysis also showed a highly significant interaction between ploidy and number. When mean numbers of stomata for 2n and 4n leaves were compared in respect to position on the leaf, it was found that in the diploid leaf the number increased from base to tip, while in the tetraploid leaf the order was reversed (table 7).

TABLE 5. Mean number of stomata for three positions on the leaves of *V. Miss Joaquim* (2n and 4n leaves combined)

Position on leaf	MEANS IN RANKED ORDER		
	BASE	CENTER	TIP
Mean number of stomata	6.50	6.93	7.87

Note: Means not underlined by the same line are significant at the 5% level.

In the test for size differences in stomata between 2n and 4n leaves, variance analysis revealed highly significant differences with 4n leaves possessing larger stomates than 2n leaves. The mean stomata size of the 4n plant was 55.14 microns, while that of the 2n plant was 47.03 microns long.

Significant value was also observed for differences in size of stomata in the different positions on the leaf. The mean sizes for the 2n and 4n stomates are presented in ranked order in table 6. In both cases, the stomata size increased from the tip to the base.

TABLE 6. Means of stomata size for four positions on the leaves of 2n and 4n plant

Position on leaf	MEANS IN RANKED ORDER			
	TIP	CENTER	BASE	SHEATH
Mean size in microns (2n)	41.12	46.48	46.57	53.96
	TIP	CENTER	SHEATH	BASE
Mean size in microns (4n)	51.86	53.18	56.86	58.66

Note: Means not underlined by the same line are significantly different at the 5% level.

The number and size of stomata at the various positions on the leaf showed some interesting relationships (table 7). In the case of the 2n leaf, the number and size of stomata appeared to be inversely correlated, while the relationship in the case of the 4n leaf was a positive one. A straight parallel relationship was somewhat disrupted by a slight reduction in number of stomata at the central position.

TABLE 7. Relationship of number and size of stomata according to position on the leaf for 2n and 4n plant

		TIP	CENTER	BASE
Diploid leaf:	Mean number	8.77	7.30	5.42
	Mean size (microns)	41.12	46.48	46.57
Tetraploid leaf:	Mean number	6.97	6.55	7.57
	Mean size (microns)	51.86	43.18	58.66

DISCUSSION AND CONCLUSIONS

Although treating seeds with colchicine was found to be an effective method of doubling the chromosome numbers in many plants, it was not applicable to orchids under the conditions of the experiments reported here. A probable reason is that orchid seeds are relatively slow in germination and the period of soaking may not have been long enough for the seeds to begin active growth. Another possible reason is the extreme sensitivity to colchicine injury due to lack of substantial protection of the embryo.

Seedling treatment methods reported to be highly effective by Dermen and Darrow (*11*), Newcomer (*31*), and Braak and Zeilinga (*3*) were also employed on *Dendrobium* seedlings without success. In all cases seedlings were seriously injured with high mortality rates. Rotor (*33*) reported successful chromosome doubling with *Cattleya* and *Phalaenopsis* seedlings by immersing in aqueous solutions of colchicine. However, he did not provide cytological evidences of induced doubling. No information relative to concentrations or durations of exposure was given for the reported success in doubling of chromosomes.

Because of the slow rate of growth and sensitive nature of orchid seedlings, a less drastic treatment over a longer duration may be more effective. A few drops of aqueous or glycerin-colchicine applied to the apex of the seedlings over several days or even weeks might be effective. This method appears to be suitable for *Dendrobium* seedlings because they possess natural pockets formed by the leaves at the terminal. However, the apical meristem is surrounded by layers of leaf sheath which may impede the penetration of colchicine.

Treatment of inflorescences also produced negative results. The premature yellowing and abscission of buds reported by Rotor (*33*) for *Cattleya* and *Phalaenopsis* were also observed. Concentrations lower than 0.1 percent over 2 to 3 days may be more effective than those which were used in the experiments reported here. Time

of application may be a factor to consider because the colchicine must be applied when pollen mother cells are in a rapid state of division in the production of spores.

Tip cuttings and excised shoots immersed in aqueous-colchicine for several days induced some polyploids. Similar treatment for plants first established on tree-fern poles appeared to be promising. Even though a few polyploids were produced by these methods, the low frequency indicates induction by chance and no definite percentage success can be predicted for these methods. Apparently, there must exist some favorable physiological condition besides the cytological condition of rapid cell division necessary for doubling to take place. Defining these physiological conditions remains an interesting research problem.

The lanolin and glycerine-colchicine applications on incised apices produced negative results. Incision was too drastic a treatment for the apex, causing injury to the apical dome. In most instances further growth of the apical meristem was arrested and shoots from axillary apices emerged. These new shoots were all determined to be diploids.

Although *Dendrobium* and spatulate-leaf *Vanda*, by the nature of their leaf shape and position of leaves, appear to afford a good area at the tips of the plant for injection of colchicine solutions, the deeply embedded apical dome makes application difficult. The high mortality rates in *Dendrobiums*, including the check group (injected with water), indicated physical injury by the capillary pipettes injected into that area.

In evaluating the various methods, it was found that basal treatment of cuttings and shoots in colchicine concentrations between 0.5 and 1.5 percent for durations of 2 to 6 days gave the best results. Lower concentrations at longer durations were not effective, probably due to injury of the cells in the immersed section of the stem. Brown discoloration was observed in the immersed tissues. Similar injuries were noted in immersed stem sections in high concentrations of colchicine. Such injuries could prevent further absorption of colchicine solution.

Within the effective range of concentration and duration, a large number of cuttings or shoots must be treated because doubling appears to be by chance. Furthermore, this method is conducive only to orchids with monopodial growth habits like that of *Vanda*. Even with *Vanda*, the terete and semi-terete types seem to be more suited to this method than the strap-leaf type because of the elongated stems of the former two types.

The temporary and mixed nature of polyploidy and diploidy found in this study indicates the presence of sectorial chimeras and mixoploids, much like those reported by Satina, *et al.* (*35*), Satina and Blakeslee (*34*), Dermen (*8*), and others. Analysis of these chimeras and mixoploids and their uses in the study of ontogeny and histogenesis in orchids will be reported in a subsequent paper in greater detail.

The sizes of cells as shown in the photomicrographs are different between 4n and 2n tissues. Cells in the 4n tissues are visibly larger than those of the 2n counterparts. The gigas condition exhibited by orchid polyploids is probably due to increase in cell size.

Although stomatal numbers have been found to be the same between 2n and 4n plants, there were differences in number in the various positions on the leaf.

This quantitative relationship between number and position is an inverse one when the mean numbers of 4n and 2n leaves are compared. The cause of this inverse relationship is not known.

The larger stomata on the tetraploid leaf is in agreement with increased size of cells in the tunica layer of the shoot apex. The size of stomata was found to be significantly different at the various positions on the leaf, even in the diploid plant. The inverse relationship between number and size of stomata at the various positions on the 2n leaf was as expected, i.e., decreasing size with increasing number per unit area. However, no immediate explanation can be given for the positive relationship observed in the case of the 4n leaf.

Such information becomes important when stomatal size is used to determine ploidy. The data in table 6 show that stomatal size in the epidermis of the 2n sheath is similar to those found in the central portion of the 4n leaf. The difference in size is also reduced when stomates at the tip of the 4n leaf are compared with those at the base of the 2n leaf.

SUMMARY

To determine feasible methods of doubling the chromosome numbers in seeds, seedlings, young shoots, stem cuttings, and mature plants, several *Vanda* and *Dendrobium* species and hybrids were treated in various ways using different concentrations of colchicine in carriers such as water, glycerine, nutrient agar, and lanolin. Seeds were soaked in colchicine solution before sowing or sowed directly in colchicine-incorporated nutrient agar. Seedlings were soaked in various concentrations of aqueous-colchicine. One lot of seedlings was soaked in colchicine under vacuum. Cuttings and young shoots were treated by immersing the basal ends in aqueous-colchicine for different durations. Lanolin-colchicine and glycerine-colchicine were applied into incised areas in the apical regions of the plants.

When the results were evaluated, it was found that tetraploidy was induced only in cuttings and young shoots of *Vanda Miss Joaquim* treated by immersing the basal ends in aqueous-colchicine. Effective concentrations were between 0.5 and 1.5 percent, and durations of exposure between 2 and 6 days. Within these concentrations and durations, successful doubling still remained a matter of chance.

Among the 10 induced tetraploids, only one gave 4n number consistently at all nodes tested. Seven were temporary in nature with reversion to diploidy after the first few 4n counts were made. Two plants gave variable numbers of 4n and 2n in the same roots or in different roots at succeeding nodes. These variable counts indicate the production of cytochimeras and mixoploids.

The size of the cells in the tetraploids was visibly larger than those of the diploids. Stomatal number was determined to be the same for 2n and 4n leaves, but size differences were highly significant with the 4n leaves having larger stomates. The relationship between number and size of stomates with respect to position on the diploid leaf was negative while that of the tetraploid leaf was a positive one.

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